# Surface-enhanced infrared absorption-reflectance (SEIRA) microspectroscopy for bacteria localization on geologic material surfaces

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## Abstract

Surface-enhanced infrared absorption-reflectance (SEIRA) microspectroscopy is potentially a useful chemical/biological probe to provide insights into the localization of living endolithic bacteria on the surfaces of geologic materials. This hypothesis was tested by validating and demonstrating the use of SEIRA with a metal-overlayer configuration to identify qualitatively on vesicular basalt surfaces the highly localized differences in the chemical composition and in the structure of clusters of endolithic bacteria, vesicles, and minerals. The metal-overlayer configuration was achieved by evaporating a thin gold-film on basalt specimen surfaces. Fourier-transform SEIRA microspectra of the specimen surfaces were recorded in the $650–4000 \text{ cm}^{-1}$ infrared region at a resolution of $4 \text{ cm}^{-1}$ on a Fourier-transform infrared spectrometer coupled to an infrared microscope. All bacteria-inhabiting surfaces exhibited infrared absorption bands indicative of bacterial cells, bands that became ideal biomarkers by which to detect the presence of bacteria. All basalt surfaces exhibited infrared absorption bands indicative of silicates, bands that became ideal mineral markers by which to detect the presence of silicate-containing minerals and locations of vesicles (gas-bubble cavities). Comparative analysis of space-resolved microspectra suggested that bacteria in the vesicular basalt lived not only on the vesicle surface but that they also penetrated and lived beneath the vesicle surface. The penetration terminated when calcic-plagioclase feldspar became the dominant constituent mineral in the vesicular basalt. With this experimental effort, the practical aspects and the usefulness of SEIRA as a promising tool to complement existing techniques for studying the in-situ localization of living bacteria in geologic materials have been demonstrated. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Endoliths; Basalt; Minerals; Infrared spectroscopy; Infrared microspectroscopy

## 1. Introduction

Pollution of subsurface geologic zones and the possibility of using the intrinsic endolithic (rock/mineral-inhabiting) bacteria to either detoxify or immobilize the pollutants have stimulated new inter-

ests in the exploration of endolithic bacteria and their long-term survival in the geologic environment. The location of bacteria within rocks and minerals affects the type and intensity of environmental stresses the bacteria are exposed to and thus affect their long-term survival potential. Localization of bacteria within rocks has been the subject of many ongoing research programs Ferris and Lowson, 1997; Fredrickson et al., 1997. Two strategies are commonly used

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to answer the question of relationships between the microbial localization and the microstructure of rocks. The first approach is a microscopic system approach Ferris and Lowson, 1997. It uses microscopy techniques to visually enumerate the distribution of endolithic microorganisms on an intact environmental sample. The distribution is related to the microscale physical and geochemical features measured on the same intact environmental sample. The other approach is a global system approach Fredrickson et al., 1997, which uses molecular and/or metabolic probes to identify the presence of microorganisms in crushed rocks and conventional methods to identify the physical and geochemical properties of the rock materials.

In this paper we present the use of surface-enhanced infrared absorption-reflectance (SEIRA) microspectroscopy as a complement to the above techniques to qualitatively study relationships between the microbial localization and the microstructure of geologic materials such as rocks. SEIRA microspectroscopy combines the traditional light-reflecting microscopy with the more recent but well-documented SEIRA spectroscopy technique. The fundamental working concept of SEIRA microspectroscopy is to use visible light and reflecting optics to view a magnified image of the sample and to select a microscopic surface area on the sample for infrared reflection-absorption spectroscopic analysis Reffner and Martoglio, 1995. The selection of the area is relatively subjective and relies on the geometry, color, crystallographic properties, and other material-specific features of the sample surface. Once the sample area is selected, the molecular information of the selected surface area can be recorded in-situ spectroscopically in the infrared spectral region. For samples with surfaces of low infrared reflection, such as rock surfaces Hofmeister, 1995; Kellner et al., 1997, or with low analyte concentrations, such as those typically encountered in studies of environmental pollution Kellner et al., 1997, the measured spectra often do not have sufficient sensitivity for obtaining molecular information of the surface. Hartstein et al. Hartstein et al., 1980 and Hatta et al., 1984 found that SEIRA spectroscopy using a metal-overlayer or metal-underlayer configuration could tremendously increase the spectral sensitivity of molecules that are either in contact with or near the

metal film. With the appropriate metal-layer configuration, especially in the presence of well-defined clusters of metal-islands Kellner et al., 1997, many researchers demonstrated that the sensitivity of the enhanced reflection infrared spectra could be increased by up to three orders of magnitude Kellner et al., 1997; Hatta et al., 1984; Osawa et al., 1993. The mechanism of the increase is still a controversial matter, but it is generally believed to arise from the enhancement of the incident infrared field at the metal surface. Recent literature has demonstrated that SEIRA spectroscopy has been successfully used in a wide variety of analyses for water films Ataka et al., 1996 and thin organic films on semi-conductors, glasses, and polymer Hatta et al., 1984; Osawa et al., 1993; Johnson and Aroca, 1995; Wanzenböck et al., 1997; Johnson et al., 1997; Nishikawa et al., 1993.

We hypothesized in this study that SEIRA microspectroscopy, using a metal-overlayer configuration (Fig. 1), can be used as a chemical/biological probe to monitor the microbial localization on surfaces of geologic materials. Although geologic materials inherently have low infrared reflective surfaces, their intrinsic surface roughness can function as well-defined clusters of metal-islands once they are coated with thin films of metals and thus increase the success of the method. The fundamental purpose of this study was to test the above hypothesis by validating and demonstrating the use of this technique to identify qualitatively on vesicular basalt surfaces the highly localized differences in the chemical composition and structure of clusters of intrinsic endolithic bacteria, vesicles, and minerals. This study not only demonstrates the usefulness of the SEIRA microspectroscopy technique but also provides results that will shed new light on the
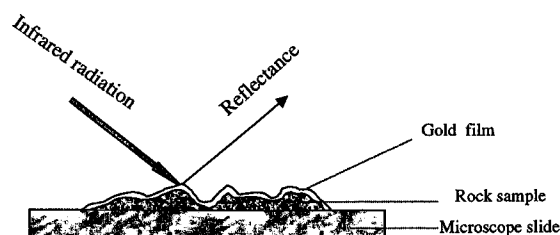


Fig. 1. A schematic diagram of the metal-overlayer configuration.

significant factors responsible for the localization of bacteria in geologic materials such as rocks.

## 2. Experimental

### 2.1. Sample collection, handling, and preparation

Vesicular basalt cores (8.6-cm diameter) were collected from a subsurface rock vadose zone at a depth of 66–70 m at a site within the extensive Columbia basalt flow in southeastern Idaho. The sampling technique, sample handling, and sectioning at the drilling site were conducted according to the protocol developed under the U.S. Department of Energy Subsurface Science Program specifically to minimize disturbances to intrinsic microbial communities in the sample Griffin et al., 1997. Earlier analysis Sorenson et al., 1996 showed that vesicular basalt at the site were fine-grained silicate-containing rocks, with calcic-plagioclase feldspar (Na, Ca) $(AlSi)_4O_4$, pyroxene (Ca, Na, Mg, Fe) (Al, Si) $O_3$, and olivine $Mg_{1.8}Fe_{0.2}SiO_4$ being the essential minerals. About 10–25% of the matrix consisted of vesicles of various sizes. Some vesicles were partly infilled with individual or mixtures of minerals.

Sectioned cores were transferred at the site into Whirl-pak bags and placed in sterile, argon-filled canisters, sealed, and shipped under Blue Ice by overnight express to our laboratory. Upon arrival, the cores were opened aseptically inside a sterile laminar hood to avoid contamination of samples for future microscopic and microbiological analysis. To minimize the possibility of future contamination from airborne microorganisms, all unused rock samples were immediately placed in sterile plastic bags and transferred to sterile gas-tight containers filled with air for storage at 4°C until used.

Specimens of 40- to 100-μm thickness were prepared inside a sterile laminar hood by cleaving basalt fragments off the micro-fissile near the center of the rock samples. It was assumed that the center of the rock sample was unlikely to suffer any ex-situ bacterial contamination from sample collection and handling processes. The cleaved basalt specimens were used to demonstrate the utility of SEIRA microspectroscopy for qualitatively identifying on the cleavage surface the highly localized differences

in the chemical composition and structure of clusters of endolithic bacteria, vesicles, and minerals. They were stored at 4°C until used. Meanwhile, bacteria on the corresponding 'exposed' surface of the cleaved rock sample were cultivated, purified, and used to validate the SEIRA microspectroscopy technique.

### 2.2. Minerals

Calcic-plagioclase feldspar, pyroxene, and olivine, the essential mineral composition of the vesicular basalt samples, were purchased from Minerals Unlimited (Ridgecrest, CA). Specimens of 40- to 100-μm thickness were prepared from these mineral samples. Together with a selection of vesicular basalt specimens prepared earlier, their surfaces were cleaned for 15 min by sonication in deionized and organic-free water before they were used to provide reference spectral information and to validate the SEIRA microspectroscopy technique.

### 2.3. Culturing and profiling of intrinsic endolithic bacteria

The 'exposed' surface of the cleaved rock sample was subject to an agar printing-off procedure Hirsch et al., 1995 to identify the intrinsic bacteria that could be cultivated and to select bacteria-containing specimens for the SEIRA experiments. The agar media were the basalt extract-yeast extract agar which consisted of 60% distilled and de-ionized water, 40% filter-sterile basalt-extract solution, 22.2 g/l of agar, 0.1 g/l each of Bacto yeast extract and glucose. The basalt extract solution was prepared by mixing vigorously and heating (without boiling) for 1 h 500 g of basalt grains in 1 l of distilled, de-ionized water. The liquid was strained through a double-layer cheesecloth and filter sterilized with 0.2 μ CA/CN (cellulose acetate/cellulose nitrate mixed esters) filters. The printing-off procedure involved transferring the rock sample with the 'exposed' surface upside down onto the agar and pressing against it for 1 min while avoiding dislocation. The 'inoculated' agar disc was incubated at room temperature ($\approx 21$°C) in the dark for 5 days. Colony development on the 'inoculated' agar disc was checked daily.

Individual colonies developed on the 'inoculated' agar disc were purified by streak plating onto the basalt extract-yeast extract agar. The purity of the isolates was confirmed by microscopic inspection of the colony morphologies of the isolate colonies and Gram-staining. Pure-culture plates were achieved when all isolated colonies had the same colony morphology, and the cell morphology of an isolated colony appeared uniform in a Gram-stain. Their identity determination was based on colony and cellular morphology and fatty acid methyl ester (FAME) analysis. The log-phase living cells of purified isolates were used to validate the SEIRA microspectroscopy technique. The log-phase living cells were obtained by re-culturing the purified isolates individually in 100 ml of sterile basalt extract-yeast extract using 1000-ml flasks fitted with cotton plugs. Bacteria cells were incubated at room temperature for 2 days with continuous stirring at 250 rpm. Cells were concentrated and cleaned by centrifugation at 5000 g for 10 min and washed with and re-suspended in 10 ml of mineral salt medium three times.

## 2.4. Validation of the use of SEIRA microspectroscopy

To achieve the goal of validating the SEIRA microspectroscopy technique, Fourier-transform SEIRA microspectra were obtained for films of log-phase bacterial cells on aluminized microscope slides, on clean basalt cleavage surfaces, and for the clean cleavage surfaces of calcic-plagioclase feldspar, pyroxene, olivine, and basalt. Bacterial films on surfaces were produced by introducing drops of concentrated bacteria suspension using disposable pipettes. They were allowed to dry in sterile desiccators overnight at room temperature. Samples of bacteria films on aluminized microscope slides and on vesicular basalt surfaces, as well as the clean mineral and vesicular basalt specimens, were dried in the same manner.

Shortly before the microspectroscopic experiments, all samples were further air-dried for up to 20 min inside the laminar hood (Sterilgard II, Type A/B3, Baker Company) to ensure that the residual water trapped in the surface of the samples was adequately removed. That the 20 min drying was

sufficient to drive off the residual water from the specimen surfaces was confirmed when water vapor absorption bands and their second derivative bands in the $1800–1500$ $cm^{-1}$ region did not exhibit detectable changes with increasing drying time. To obtain a metal-overlayer configuration (as shown schematically in Fig. 1), the air-dried specimens were immediately affixed to an aluminum stub with a double-sided tape and gold-coated in a Polaron sputter coating system at a current of 20 mA and a pressure of 0.06 Torr. Our earlier work has shown that for a biogeochemical specimen such as bacterial films on basalt surfaces, the sensitivity of the enhanced infrared spectra depends on the length of the coating time (i.e., the thickness of the gold film). In this study we coated the specimen for different incremental time for up to 80 s. The corresponding Fourier-transform SEIRA microspectra were recorded and compared. Results were used to determine the appropriate coating time that would produce the highest signal enhancement for samples with similar surface characteristics.

All Fourier-transform SEIRA microspectra were recorded in the $4000–650$ $cm^{-1}$ infrared region on a Nicolet Model 750 FTIR-spectrometer coupled to an IR-Plan microscope. The $4000–650$ $cm^{-1}$ infrared region was selected, because it is the region that contains unique molecular fingerprint-exhibiting absorption features of intact bacterial cells Carr et al., 1995; Brandenburg and Seydel, 1996; Naumann et al., 1996 and silicate-containing basalt and its essential minerals Salisbury et al., 1991; Hunt, 1989. For each infrared measurement, 128 spectra were co-added at a spectral resolution of 4 $cm^{-1}$. All infrared spectra were ratioed against the spectra of gold-coated microscope slides to produce absorbance values. The spectral characteristics were compared to well-documented spectral research literature.

## 2.5. SEIRA microspectroscopy for spatial distribution of microorganisms

Once the technique of SEIRA microspectroscopy with a metal-overlayer configuration was validated, we conducted two mapping experiments to demonstrate its utility as a tool to identify qualitatively on vesicular basalt cleavage surfaces the highly localized differences in the chemical composition and

structure of clusters of endolithic bacteria, vesicles, and minerals. This involved combining the SEIRA microspectroscopy with confocal laser scanning microscopy (CLAM). We first selected one of the bacteria-containing cleaved basalt specimens. A Nikon molecular dynamics confocal laser-scanning microscope with fluorescence objectives was used to visually examine and record locations of bacteria on the specimen surface under simultaneous excitation at 488 and 510 nm. Thereafter the specimen was air-dried using the aforementioned procedure and treated with a metal-overlayer configuration for the SEIRA microspectroscopy mapping measurements.

All space-resolved Fourier-transform SEIRA microspectra of the cleavage surfaces were again recorded on the same FTIR spectrometer – IR-Plan microscope system but equipped additionally with a computer-controlled $x$–$y$ mapping stage. With the automatic step-by-step collection of microspectra, maps that showed the absorption peak height of a functional group of molecules vs. the physical location on the sample surface were obtained. For each measurement at a given location, 128 spectra were again co-added at a spectral resolution of 4 cm$^{-1}$. To eliminate the distortion of the recorded image, we ensured an overlapping of the sampling spot by choosing the step-width in both $x$ and $y$ directions to be half of the diameter of the aperture set on the infrared microscope, as has been demonstrated by Mizaikoff et al., 1993.

## 3. Results and discussion

### 3.1. Profiles of cultivable endolithic bacteria

Six morphologically distinct colonies were recovered from the indigenous bacteria on the 'exposed' surface of the cleaved rock sample. Two were Gram-negative (*Pseudomonas putida*, and *Pseudomonas fluorescens*) and four were Gram-positive (*Arthrobacter oxydana*, *Bacillus atrosepticus*, *Micrococcus lylae*, and *Nocardia globerula*). Similar bacteria have been found in other terrestrial deep subsurface environments Haldeman et al., 1993; Amy, 1997; Balkwill and Boone, 1997. Living cells from the Gram-negative colonies fluoresced naturally and produced yellow–green to blue–green color under

simultaneous excitation at 488 and 510 nm; living cells from the Gram-positive colony also fluoresced naturally and produced a pale yellow color.

### 3.2. Validation of SEIRA microspectroscopy

The global spectral features of our Fourier-transform SEIRA microspectra for the six endolithic bacteria isolates are consistent with those reported in the literature Brandenburg and Seydel, 1996; Naumann et al., 1996; Naumann, 1984; Nichols et al., 1985; Fragata et al., 1993; Naumann et al., 1982, 1991a, 1991, 1994; Zeroual et al., 1994; Nivens et al., 1993; Brock et al., 1994; Taga et al., 1994; Jackson and Mantsch, 1996; Kennedy et al., 1991; Mantsch et al., 1993; Torii and Tasumi, 1996; Lipkus et al., 1990. All have well-documented prominent absorption envelopes arising from the C=O functional group of biomolecules: the stretching vibrations in the 1695–1620 cm$^{-1}$ region of protein Amide I with C=O weakly coupled to the N–H stretching mode, and the bending vibrations in the 1570–1515 cm$^{-1}$ region of protein Amide II with C=O strongly coupled to the N–H stretching mode. Gram-negative isolates exhibit an additional but relatively weak ester carbonyl peak near 1741 cm$^{-1}$, which arises from the C=O ester stretching vibration of phospholipids in their additional outer membrane.

The global features of the SEIRA microspectra of the clean and bacteria-free basalt cleavage surfaces agree with those reported for silicate-containing rocks and minerals in the literature Salisbury et al., 1991; Kahle and Goetz, 1983; Walter and Salisbury, 1989; Hunt, 1989. These spectral features differed depending on the constituent minerals at the surface within the sampling area. In some cases the spectrum was an anomalously bland basalt spectrum that consisted of contributions from the multiple constituent minerals. In other cases the spectral features became sharp and intense and were similar to the spectral features from individual (or a mixture of fewer) minerals. In Fig. 2 an example of the SEIRA microspectra of a basalt cleavage surface and its major constituent minerals for comparisons are shown. The SEIRA microspectrum of the basalt surface has intense spectral features in the 1300–800 cm$^{-1}$ region. They arise predominantly from fundamental asymmetric Si–O–Si and Si–O–Al stretch-
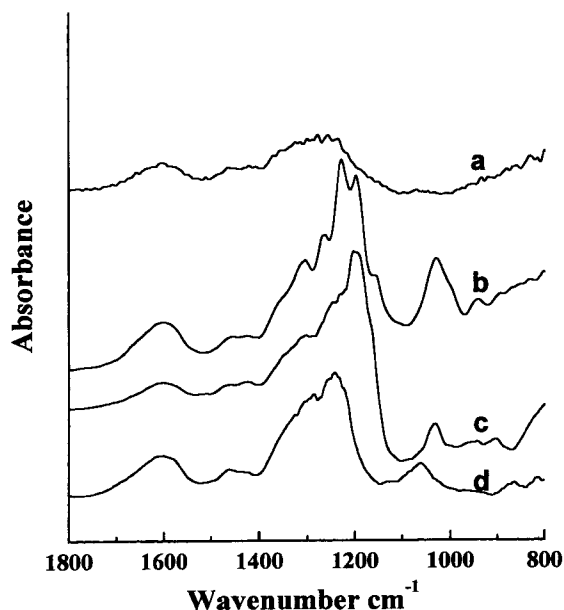
Fig. 2. SEIRA absorption spectra of clean and bacteria-free cleavage surfaces of (a) vesicular basalt, (b) calcic-plagioclase feldspar, (c) pyroxene, and (d) olivine. All spectra are shown by scaling the strongest bands to be equal. (Aperture = $50 \times 50$ μm).

ing vibrations of the silicates in basalt and have been widely used as a terrestrial marker for different silicate-containing rocks and minerals Salisbury et al., 1991; Kahle and Goetz, 1983; Walter and Salisbury, 1989. The minor absorption band centered around 1640 cm$^{-1}$ has been assigned to the H–O–H bending vibration. The assignment of the weaker bands centered around 1430 cm$^{-1}$ is less certain, but they are believed to be the overtone/combination bands of the silicate group Salisbury et al., 1991.

The SEIRA microspectra of cleavage surfaces of calcic-plagioclase feldspar, pyroxene, and olivine have very different spectral features in the 1300–1000 cm$^{-1}$ region. Although the spectrum of a vesicular basalt cleavage surface cannot simply be generated by adding the constituent mineral spectra in the proportions in which they occur in the specimen, spectral information of individual silicate-containing minerals can be helpful to qualitatively identify minerals in a specimen with a mixture of two silicate-containing minerals Hunt, 1989.

In Fig. 3 the SEIRA microspectra recorded for two similar basalt surfaces: one with and one without a film of Gram-negative bacteria are compared. The overall spectral features of the bacteria-containing basalt surface agree with those of the bacteria-free basalt surface; except for the absorption peak at 1741 cm$^{-1}$ of phospholipids and a series of relatively well-defined peaks in the 1700–1500 cm$^{-1}$ of the protein Amide I and II regions. The extent of signal enhancement of these peaks appears to be related to the gold-coating time, as shown in the insert in Fig. 3 obtained for another basalt surface. From 30 to 60 s of coating time there was a persistent increase in spectral enhancement with longer coating times (i.e., with an increasing thickness of the gold film). In addition, the signal enhancement was mainly observed for the well-defined absorption peaks in the 1700–1500 cm$^{-1}$ of the protein Amide I and II regions. This selective enhancement implies that there is a preferential increase in the sensitivity of the infrared absorption spectrum, which is likely due to the physical and chemical properties of the sample surface and the SEIRA technique. Similar selective enhancement has been reported Kellner et al., 1997; Hatta et al., 1984; Osawa et al., 1993.

Results from the above analysis of the SEIRA microspectra for materials in our biogeochemical experimental system indicate that the following distinct SEIRA absorption bands should be used as biomolecule markers (biomarkers) to detect spatial distribution of bacteria microcolonies on the basalt specimen surfaces: the C=O ester carbonyl peak near 1741 cm$^{-1}$, the C=O Amide I peaks (within the Amide I envelope) near 1650 cm$^{-1}$, and the Amide II peaks (within the Amide II envelope) near 1550 cm$^{-1}$. The broad features of the absorption spectra for the constituent minerals in the region between 1300 and 800 cm$^{-1}$ were used to identify the distribution of vesicles in the basalt specimens. Detailed changes in the spectral features in the 1300 and 1000 cm$^{-1}$ regions were also used as mineral markers to identify changes in the constituent minerals, although at times this approach of identification could become difficult and less certain in our multimineralic specimen.

### 3.3. SEIRA microspectroscopy for spatial distribution of microorganisms

Two SEIRA microspectroscopic mapping experiments were conducted on the selected specimen to
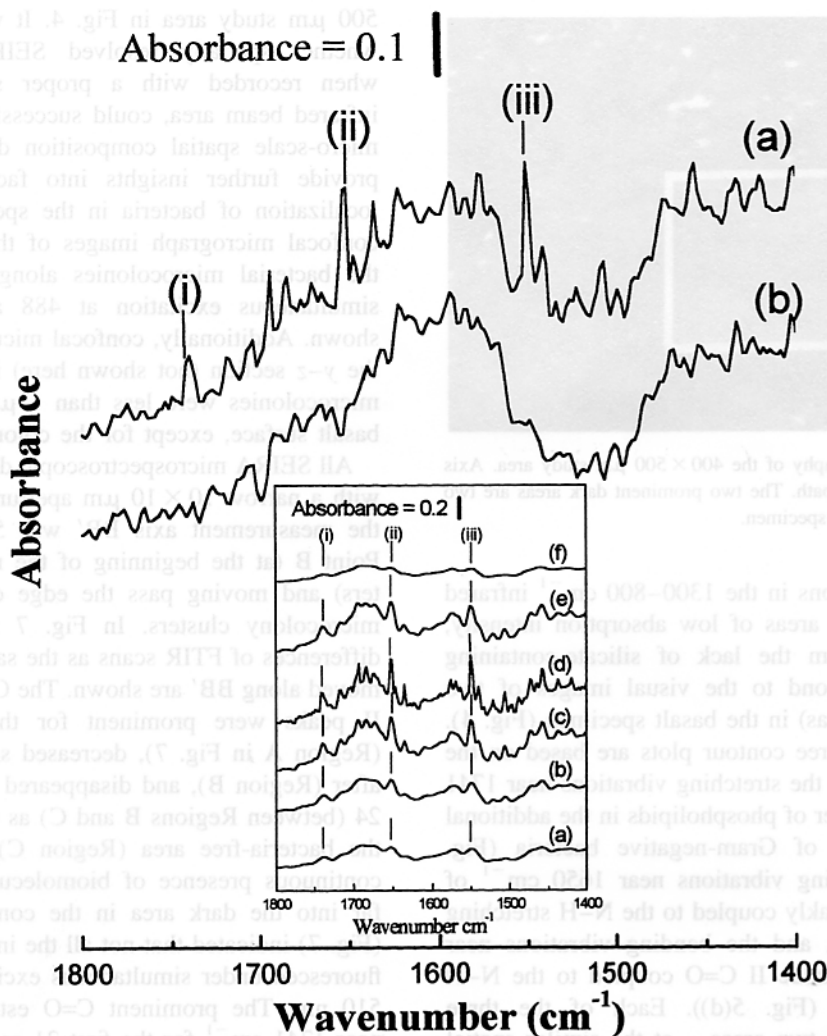
Fig. 3. SEIRA absorption spectra of basalt cleavage surfaces (a) with and (b) without a bacterial film. The three distinct enhanced biomarker peaks are near (i) 1741 cm$^{-1}$ of phospholipids in the outer membrane of Gram-negative bacteria, (ii) 1650 cm$^{-1}$ within bacterial protein Amide I envelope, and (iii) 1550 cm$^{-1}$ within bacterial protein Amide II envelope. Insert shows the variation of sensitivity of the biomarker peaks with gold-coating time (a) 30 s, (b) 40 s, (c) 50 s, (d) 60 s, (e) 70 s, and (f) 80 s. All spectra are shown by scaling the strongest bands to be equal. (Aperture = 50 × 50 μm).

demonstrate the potential utility of SEIRA microspectroscopy as a chemical/biological probe to relate the localization of living endolithic bacteria to the physical and geochemical characteristics of the rock surface. The first experiment was to apply SEIRA microspectroscopy to study the relation of the localization of intrinsic endolithic bacteria and the location of vesicles within the basalt specimen. The study area covered 400 × 500 μm (Fig. 4) and was ir-

radiated with an infrared beam with a diameter of 100 μm. The step width in both the $x$ and $y$ directions was 50 μm.

In Fig. 5 the resulting contour plots of absorbance of the study area are shown. The first contour plot (Fig. 5(a)) represents the distribution of vesicles on the specimen surface, which is based on the absorption from the mineral silicate functional groups (fundamental asymmetric Si–O–Si and Si–O–Al
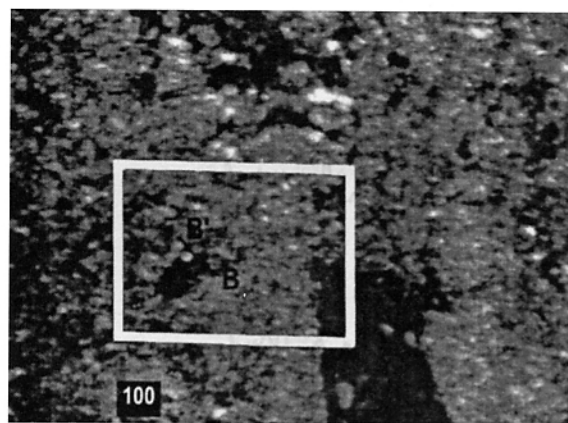
Fig. 4. Microphotography of the $400 \times 500$ μm study area. Axis BB' is the sampling path. The two prominent dark areas are two vesicles in the basalt specimen.

stretching vibrations in the 1300–800 cm$^{-1}$ infrared region). The two areas of low absorption intensity, which arises from the lack of silicate-containing minerals, correspond to the visual images of two vesicles (dark areas) in the basalt specimen (Fig. 4). The remaining three contour plots are based on the three biomarkers: the stretching vibrations near 1741 cm$^{-1}$ of C=O ester of phospholipids in the additional outer membrane of Gram-negative bacteria (Fig. 5(b)), the stretching vibrations near 1650 cm$^{-1}$ of Amide I C=O weakly coupled to the N–H stretching mode (Fig. 5(c)), and the bending vibrations near 1550 cm$^{-1}$ of Amide II C=O coupled to the N–H stretching mode (Fig. 5(d)). Each of the three contour maps has two areas – at the similar spatial location – of higher absorption intensity arising from different C=O vibrational modes of biomolecules. These areas of high absorption intensity, however, appear to be at locations near the edge of the two areas of the decreased absorption intensity (i.e. vesicles) in Fig. 5(a) and extended almost uni-directionally into the basalt matrix. This observation implies that, similar to the endolithic microorganisms in surface terrestrial environment above ground, the subsurface bacteria within vadose vesicular basalt tend to bore into the basalt matrix beneath the vesicle surface.

The second experiment was a line analysis conducted at a finer scale along axis BB' through a cluster of bacterial microcolonies inside the $400 \times$

500 μm study area in Fig. 4. It was to investigate whether spatially resolved SEIRA microspectra, when recorded with a proper spatial scale and infrared beam area, could successfully illustrate the micro-scale spatial composition differences and to provide further insights into factors that control localization of bacteria in the specimen. In Fig. 6 confocal micrograph images of the $x$–$y$ section of the bacterial microcolonies along axis BB' under simultaneous excitation at 488 and 510 nm are shown. Additionally, confocal micrograph images of the $y$–$z$ section (not shown here) indicate that most microcolonies were less than 1 μm thick from the basalt surface, except for the colony 'A'.

All SEIRA microspectroscopic data were collected with a narrow $10 \times 10$ μm aperture. The step along the measurement axis BB' was 5 μm, starting at Point B (at the beginning of the microcolony clusters) and moving pass the edge of the fluorescent microcolony clusters. In Fig. 7 the spectroscopic differences of FTIR scans as the sampling point was moved along BB' are shown. The C=O Amides I and II peaks were prominent for the first 21 scans (Region A in Fig. 7), decreased significantly thereafter (Region B), and disappeared abruptly by Scan 24 (between Regions B and C) as the transition into the bacteria-free area (Region C) was made. The continuous presence of biomolecules marker peaks far into the dark area in the confocal micrograph (Fig. 7) indicated that not all the indigenous bacteria fluoresced under simultaneous excitation at 488 and 510 nm. The prominent C=O ester carbonyl peak near 1741 cm$^{-1}$ for the first 21 scans (Region A of Fig. 7) implied that Gram-negative endoliths existed along that segment of line BB'. Detailed spectral changes between regions are also shown in Fig. 8.

The decrease of biomarker peaks 1741, 1650, and 1550 cm$^{-1}$ between Scans 21 and 24 (Region B) coincided with a change of SEIRA spectral features: from a spectrum with relatively bland features (Region D) to a spectrum with more intense and sharper features (Region E). The relatively bland features observed between Scans 1 and 20 (Region D), as discussed earlier, probably arose from a basalt surface with a mixture of multiple silicate-containing minerals. The more intense and sharper features that were observed between Scans 21 and 24 (Region E) occurred when the basalt surface contained a mixture
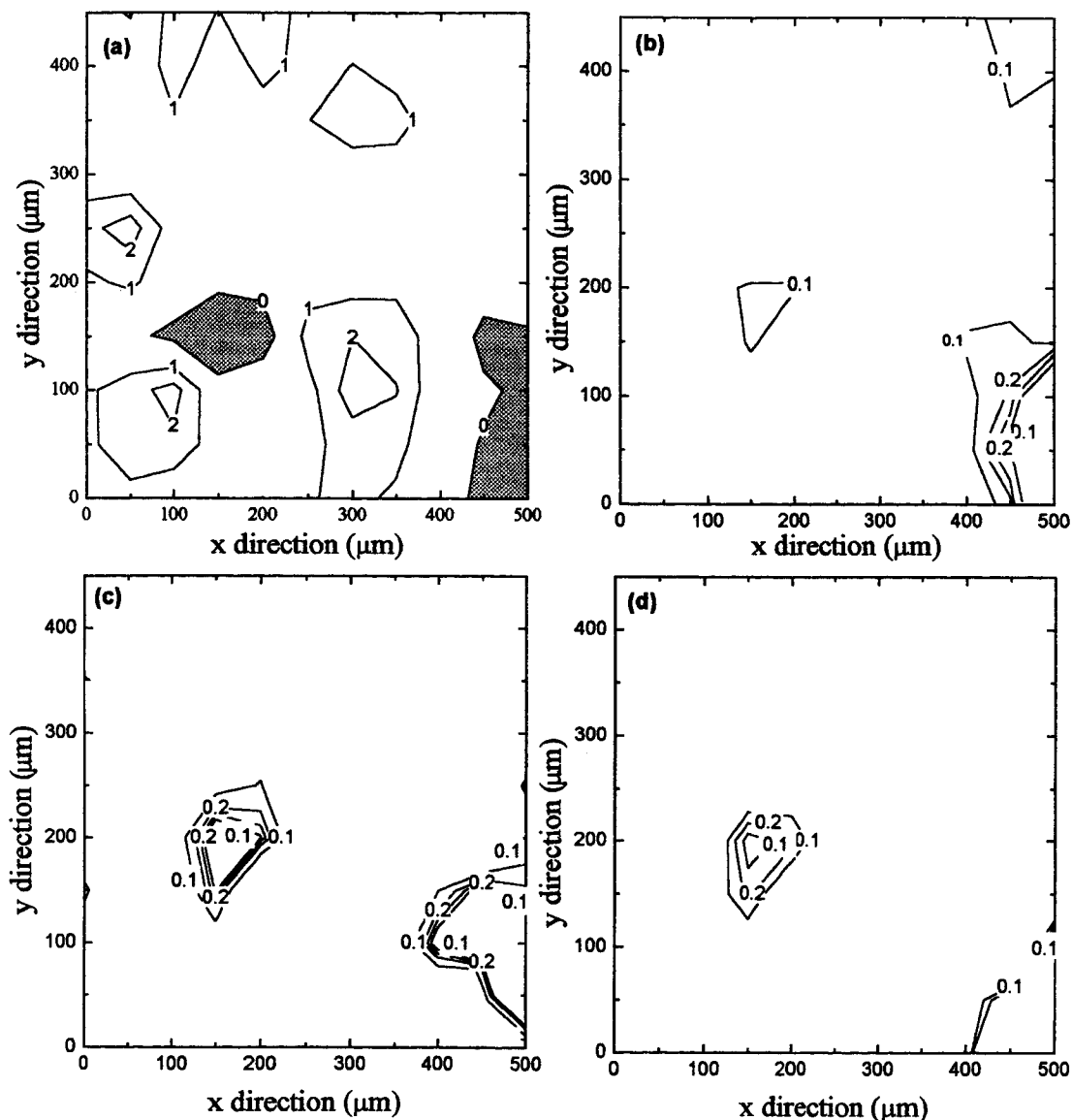
Fig. 5. Contour plots of the study area based on absorption intensity from (a) silicate-containing minerals in basalt in the 1300–800 $cm^{-1}$ region, (b) phospholipids in the outer membrane of Gram-negative bacteria at 1741 $cm^{-1}$, (c) bacterial protein Amide I at 1650 $cm^{-1}$, and (d) Amide II at 1550 $cm^{-1}$. Values denote absorbance measured with baseline adjustment. (Aperture = $50 \times 50$ μm).

of fewer silicate-containing minerals. Since rock spectra cannot be simply generated by adding the constituent mineral spectra in the proportions in which they occur in rocks, it is difficult to identify the actual shift of the major component minerals between Scans 21 and 24 (Region E). At Scan 24 (between Regions B and C) the biomarkers peaks

(1741, 1650, and 1550 $cm^{-1}$) disappeared abruptly, which coincided with an abrupt change of SEIRA spectral features: from moderately sharp features (Region E) of a basalt surface containing a mixture of fewer silicate-containing minerals to intensely sharp spectral features (Region F) that are similar to those calcic-plagioclase feldspar shown in Fig. 4 and
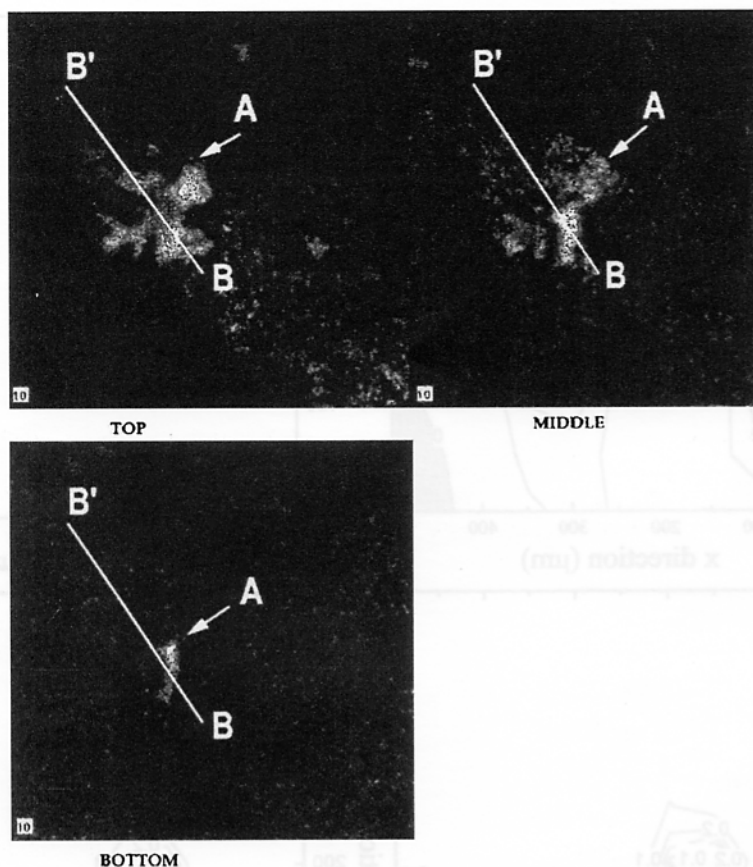
Fig. 6. Confocal fluorescence micrograph of *x*–*y* sections showing images of microbial clusters at three different optical depths on the vesicular basalt sample: 8.8 μm (top), 7.7 μm (middle), and 1.1 μm (bottom) from the base of the brightest microbial colony 'A'. The microbes were observed with simultaneous excitation at 488 and 514 nm. Axis BB′ is the same sampling path shown in Fig. 4.

again in Fig. 8 for comparison. This observation shows that the intrinsic bacterial growth terminated as calcic-plagioclase feldspar became a major component of constituent minerals in the specimen.

## 4. Conclusion

This study has demonstrated, for the first time to our knowledge, that surface-enhanced infrared absorption-reflectance (SEIRA) microspectroscopy using a metal-overlayer is a promising tool to complement existing techniques to study the in-situ localization of

bacteria within geologic materials such as rocks. The success of the technique depends significantly on the appropriate selection of the metal-coating time that would provide the sufficient signal enhancement. Within our experimental system, we have validated the technique by comparing the SEIRA microspectra to the conventional infrared spectra in the literature. We have shown that the enhanced peak near 1740 cm$^{-1}$ of the phospholipids of the Gram-negative bacterial membrane, the bacterial protein Amide I envelope centered near 1650 cm$^{-1}$, and Amide II envelope centered near 1550 cm$^{-1}$ were ideal biomarkers by which to detect spatial changes in microbial distribution. The intense features of the
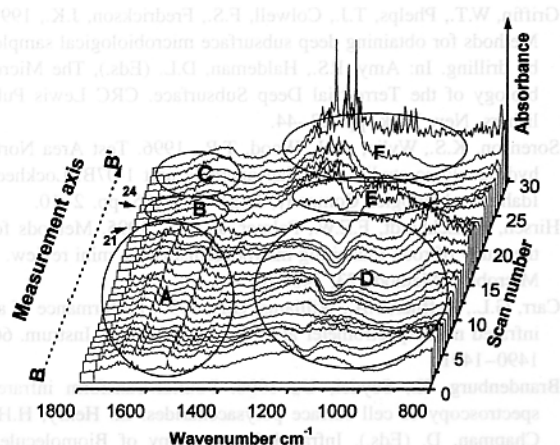
Fig. 7. A series of SEIRA spectra showing the transition from bacteria-containing to bacteria-free basalt surface along axis BB'. Each step (or scan) = 5 μm. Regions A–F are discussed in text. (Aperture = 10 × 10 μm).
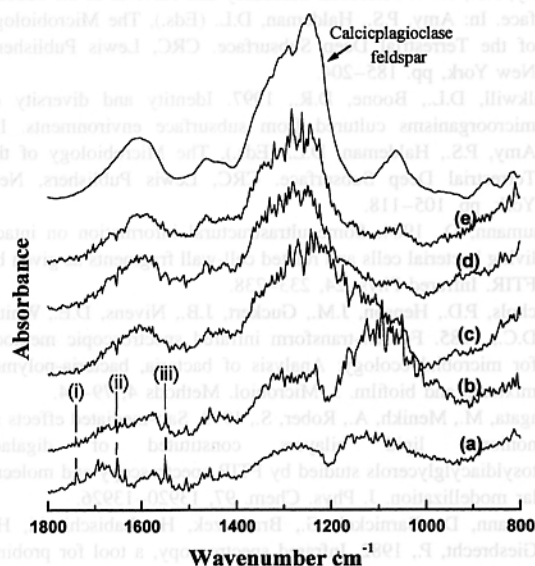


Fig. 8. Variation of SEIRA spectra (a) inside, (b) along the edge, and (c)–(e) outside the bacteria clusters. Spectra (a) = Scan 6, (b) = Scan 21, (c) = Scan 24, (d) = Scan 25, and (e) = Scan 27. (Aperture = 10 × 10 μm). Notice the general spectral similarities between spectra (c)–(e) and the calcic-plagioclase feldspar spectrum.

enhanced reflectance spectra for the constituent minerals of basalt in the region between 1300 and 800 $cm^{-1}$ were ideal marker bands for the distribution of vesicles (gas-bubble cavities) and silicate-containing minerals in the basalt specimens.

Our SEIRA microspectroscopy mapping experiments have demonstrated that SEIRA, using a metal-overlayer configuration, is a sensitive tool to complement existing techniques to study the in-situ localization of bacteria within geologic materials such as rocks. Comparative analyses of the space-resolved SEIRA microspectra from the mapping experiments suggested that bacteria in the vadose vesicular basalt not only lived on the vesicle surface but also penetrated and lived beneath the vesicle surface. Fine-scale space-resolved SEIRA microspectra showed that the penetration terminated as calcic-plagioclase feldspar became a major component of constituent minerals in the specimen.

It must be noted that the technique still has limitations. The SEIRA technique used in this study appeared to enhance the spectral sensitivity mostly in the 1800–1500 $cm^{-1}$ infrared region, which was adequate to detect the presence of bacteria within rocks and to qualitatively estimate the predominant structures of the cell protein. The sensitivity, however, is not adequate in the 1200–900 $cm^{-1}$ bacteria polysaccharide and fingerprint regions, which are important in differentiating bacteria population. Research efforts are currently underway to improve the technique.

with the molecular dynamics confocal laser scanning microscope, and Dr. R. Cowell of the Idaho National Engineering Laboratory for supplying us with the samples.

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